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Article

Biofuel that Keeps Glycerol as Monoglyceride by 1,3-Selective Ethanolysis with Pig Pancreatic Lipase Covalently Immobilized on $AlPO_4$ Support

Carlos Luna [1], Enrique Sancho [2], Diego Luna [1,*], Verónica Caballero [1], Juan Calero [1], Alejandro Posadillo [3], Cristóbal Verdugo [4], Felipa M. Bautista [1] and Antonio A. Romero [1]

[1] Department of Organic Chemistry, University of Cordoba, Campus de Rabanales, Ed. Marie Curie, 14014 Córdoba, Spain; E-Mails: qo2luduc@uco.es (C.L.); vcmvanadio@gmail.com (V.C.); p72camaj@uco.es (J.C.); qo1baruf@uco.es (F.M.B.); qo1rorea@uco.es (A.A.R.)

[2] Department of Microbiology, University of Córdoba, Campus de Rabanales, Ed. Severo Ochoa, 14014 Córdoba, Spain; E-Mail: mi1sapue@uco.es

[3] Seneca Green Catalyst S.L., Campus de Rabanales, 14014 Córdoba, Spain; E-Mail: seneca@uco.es

[4] Crystallographic Studies Laboratory, Andalusian Institute of Earth Sciences, CSIC, Avda. Las Palmeras nº4, 18100 Armilla, Granada, Spain; E-Mail: cverdugoe@lec.csic.es

* Author to whom correspondence should be addressed; E-Mail: qo1lumad@uco.es; Tel.: +34-95-7212065; Fax: +34-95-7212066.

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Abstract: By using pig pancreatic lipase (EC 3.1.1.3 or PPL) as a biocatalyst, covalently immobilized on amorphous $AlPO_4$ support, a new second generation biodiesel was obtained in the transesterification reaction of sunflower oil with ethanol. The resulting biofuel is composed of fatty acid ethyl esters and monoglycerides (FAEE/MG) blended in a 2:1 molar ratio. This novel product, which integrates glycerol as monoacylglycerols (MG) into the biofuels composition, has similar physicochemical properties as conventional biodiesel and also avoids the removal step of the by-product by washing of the biodiesel with water. Immobilization of PPL was achieved by covalent attachment of the ε-amino group of the lysine residues of PPL with the aldehyde groups of *p*-hydroxybenzaldehyde linked on a hybrid organic-inorganic functionalized $AlPO_4$ surface. With this procedure, the PPL biocatalyst was strongly fixed to the inorganic support surface (94.3%). Nevertheless, the efficiency of the immobilized enzyme was relatively lower compared to that of the free PPL, but it showed a remarkable stability as well as a great capacity of reutilization (25 reuses) without a significant loss of its initial

catalytic activity. Therefore, this enzymatic method allows the production of a biodiesel which integrates the glycerol, allows a more efficient fabrication method and minimizes the waste production as compared to the conventional alkali-catalyzed process.

Keywords: biodiesel; immobilization; pig pancreaic lipase (PPL); amorphous $AlPO_4$; selective transesterification; monoglyceride; sunflower oil

1. Introduction

It is well known that transport is almost totally dependent on fossil resources, particularly petroleum-based fuels such as gasoline, diesel, liquefied petroleum gas and compressed natural gas. As the amount of available petroleum decreases, there is a need for alternative technologies to produce liquid fuels that could potentially help prolong the usage of liquid fuels and mitigate the forthcoming effects of the increasing shortage of transportation fuels. The benefits of biofuels over traditional fuels also include greater energy security, reduced environmental impact, foreign exchange savings, and socioeconomic issues related to the rural sector. For these reasons, the share of biofuels in the automotive fuel market is expected to grow rapidly over the next decade. Biofuels could be friendly energy carriers for all countries [1]. However, the "first-generation" biofuels appear currently unsustainable, not only because of the potential stress on food commodity availability and prices, but also on the large amounts of waste generated during their production. Thus, the "second generation" biofuels ought to be built on the need for sustainable chemical products through novel green chemical technologies in order to make more simple and clean processes [2]. The "first generation" biofuels [*i.e.*, biodiesel (bio-esters), bio-ethanol, and biogas] are currently characterized either by their ability to be blended with petroleum-based fuels, combusted in existing internal combustion engines, and distributed through existing infrastructure, or by the use in existing alternative vehicle technology like FFVs ("Flexible Fuel Vehicle") or natural gas vehicles. The production of 1st generation biofuels is commercial today, with almost 50 billion liters being produced annually. However, at present, the production of such fuels is not fully cost effective, because there are a number of technical barriers that need to be overcome before their potential can be fully realized [3].

In this respect, biodiesel has attracted a lot of interest, thanks to its biodegradability, exceptional cetane numbers (as high as diesel), high flash point, low viscosity, lower combustion emission profiles, ability to be blended with fossil-based diesel at any proportions and ability to be used in conventional diesel engines with no further modifications. Biodiesel is a mixture of fatty acid alkyl esters that can be produced from several vegetable oils and animal fats via a transesterification reaction with a short chain alcohol, carried out most frequently over basic catalysts such as sodium or potassium hydroxide, methoxide, or carbonate in industries [4] or under supercritical conditions [5].

The process is normally performed under mild conditions and produces high amount of methyl esters in a short reaction time due to the formation of two phases in the reaction medium. However, a purification step is required to remove the catalyst from products and this is difficult because of its solubility in the reaction medium. This process leads to generation of large amounts of waste water containing liquids of high basicity or acidity which are not environmentally benign.

Furthermore, this homogeneous base-catalyzed system is sensitive to water and free fatty acids (FFA) that are often present in lower grade and cheaper feedstock oils, resulting in the formation of emulsions and the generation of waste water during product separation. Therefore, increased research efforts have been directed towards the development of heterogeneous catalyst systems to produce biodiesel in recent years [6]. Their benefits include simplification of the separation and purification of the reaction products, easy reuse of the catalyst in the reactor, and possibly low sensitivity to FFAs and water [7].

Enzymatic production of biodiesel has also been the subject of intensive research over the last 5–10 years [8,9]. When comparing the economy of enzymatically and chemically produced biodiesel, important factors include yield, flexibility in feedstock, value of by-products, recovery costs for alcohol as well as the cost of enzyme, enzyme lifetime, and reaction time. By 2009, large-scale enzymatic biodiesel was considered to be cost effective. Since then development work has further improved the technology, which is now ready for the market [10].

The pros and cons of using lipases as biocatalysts comparatively to alkaline and acid catalysts for biodiesel production are related to the short time and high yields obtained when chemical transesterification is applied. However, drawbacks such as high energy requirements, difficulties in the recovery of the catalyst and glycerol and potential environmental pollution are major disadvantages of the alkali or acid catalyzed processes [11,12]. In general, lipases perform their catalytic activity under more gentle conditions and with a variety of triglyceride substrates, including waste oils and fats with high levels of FFA. Furthermore, biodiesel separation and purification is much easier, resulting in a more environmentally friendly process [13,14]. However, one of the bottlenecks for industrial application of lipases is the high cost of the biocatalysts. Immobilization methods have thus been introduced to improve lipase stability and allow for repeated utilization [15,16].

Regardless of the procedure used for obtaining the conventional biodiesel, in all cases glycerol is obtained as a byproduct, representing a notable performance loss of the process given that the market is already virtually flooded by the production of glycerol, precisely obtained as a by-product in the current manufacture of biodiesel. The world forecast for glycerol production from biodiesel points to an increasing supply, with net global production around 1.2 million tons by 2012 [17], so that the excess glycerol generated may become an environmental problem, since it cannot be disposed of in the environment [18]. Thus, the transformation of waste glycerol into oxygen-containing branched compounds it is being currently considered as an interesting solution to provide an outlet for increasing glycerol stocks. In this respect, several oxygenated compounds, obtained by transformation of glycerol via etherification, esterification and acetalisation, have been assessed as additives or additional components for biodiesel formulation. The addition of these compounds has not only improved the low-temperature properties of biodiesel (*i.e.*, pour point and cold filter plugging point) and viscosity, but also did not impair other important biodiesel quality parameters analyzed. Although most of the studied oxygenated derivates do not significantly improve any biodiesel property, they do not exert a significant negative effect either. Furthermore, all of them allow an enhancement of overall yield in the biodiesel production [19].

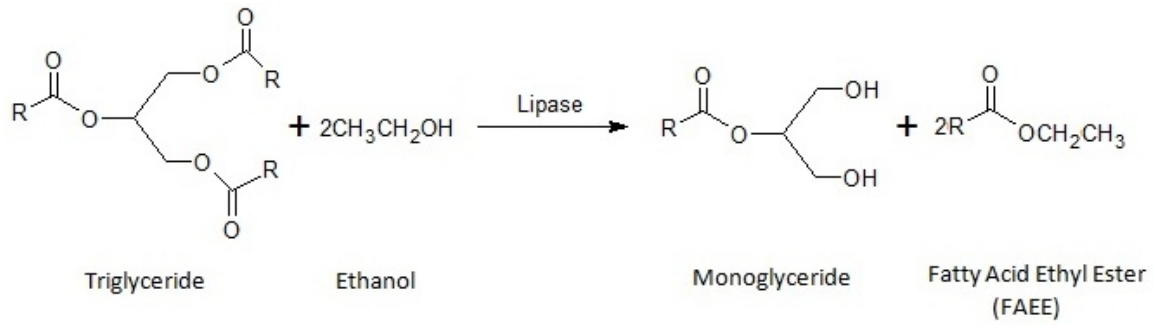
Together with this strategy, consisting in obtaining adequate oxygenated derivatives after its separation from the transesterification reaction where the glycerol is obtained, another target of great interest currently is the production, in only one reaction, of new biofuels that integrate the glycerol as a

derivative product, miscible with the FAME or FAEE obtained in a unique transesterification process. Basically, this is possible by using some alternative esters, instead of the alcohol usually employed in the conventional process.

Thus, if some glycerol derivative compound is obtained at the same time that FAME (or FAEE) in an interesterification process, a new biofuel is obtained in only one reaction. This methodology avoids the separation of glycerol before its transformation and, in a similar way as previously obtained with derivatives of glycerol [17–19], these biofuels not only prevent the generation of waste, but also increase the yields of the process, always higher than nominal 10%, by incorporating some derivatives of glycerol into the reaction products. Novel methodologies to prepare esters from lipids using different acyl acceptors which directly afford alternative co-products are currently under development [13,20–22]. The transesterification reaction of triglycerides with dimethyl carbonate (DMC) [23–27], methyl acetate [28–32] or ethyl acetate [33,34] can generate a mixture of three FAME or FAEE molecules and one of glycerol carbonate (GC) or glycerol triacetate (triacetin). These mixtures including glycerol derivative molecules have relevant physical properties to be employed as novel biofuels [35,36]. The atom efficiency is also improved as the total number of atoms involved in the reaction is part of the final mixture.

In this context, we have recently described other different protocols for the preparation of novel biofuels that integrate glycerol into their composition, consisting in the incorporation as monoglycerides (MG) of the corresponding glycerol derivatives, soluble in FAME or FAEE. In the present case, it is not necessary to appeal to any especial ester because MGs are obtained by a partial alcoholysis of triglycerides (Scheme 1). This selective process was obtained via 1,3-regiospecific enzymatic alcoholysis of sunflower oil using some 1,3-regiospecific low cost lipase, like pig pancreatic lipase, PPL [37–39] or from *Thermomyces lanuginosus* (Lipopan 50 BG from Novozymes, Bagsvaerd, Denmark) [40].

Scheme 1. Reaction scheme to obtain Ecodiesel®, a biofuel that keeps the glycerol as monoglyceride, through a 1,3-selective enzymatic ethanolysis.



As in the two methods described before [20–36], the already patented Ecodiesel-100 [37–39] obtained through the 1,3-selective partial ethanolysis of the triglycerides with PPL, is a mixture of two parts of FAEE and one part of MG, that integrates the glycerol as a soluble derivative product (MG) in the diesel fuel, but unlike these methods, no specific reagent (such as dimethyl carbonate or methyl acetate) more expensive than methanol (or ethanol) are used. This strategy is based on obtaining an incomplete alcoholysis by application of 1,3-selective lipases, so that the glycerol remains

in the form of monoglyceride which avoids the production of glycerol as a by-product, reducing the environmental impact of the process. Thus, compared to the conventional method, operating conditions are much smoother, no impurities need to be removed from the final mixture and the biofuel obtained exhibits similar physical properties to those of conventional biodiesel. In this respect, some studies have also demonstrated that minor components of biodiesel, usually considered contaminants under the biodiesel standard EN 14214, including free fatty acids and monoacyl glycerol, are essentially responsible for the lubricity of low-levels blends of biodiesel and petrodiesel. Pure FAME exhibit reduced lubricity compared to the biodiesel containing these compounds [41–45]. The presence of greater quantities of monoglycerides and/or free fatty acids enhances the lubricity of biodiesel, which is another key feature of this novel biofuel that incorporates high amounts of monoglycerides.

The actual existing limitations for the use of industrial lipases have been mainly associated with their high production costs, which can be overcome through the application of molecular technologies to achieve the production of enzymes purified in sufficiently high quantities as well as by the reuse of biocatalysts after the heterogeneous immobilization of lipases [46,47]. Here, we report the application of an enzymatic protocol using free and covalent immobilized pig pancreatic lipase (PPL) to produce the 1,3-selective alcoholysis of sunflower oil with ethanol and other short chain alcohols. In this respect, in contrast to most studied lipases, the role played by the presence of minor amounts of NaOH water solutions in the activity improvement of the enzymatic process was noticeable in our reported methodology using PPL [37–39]. PPL is an extracellular lipase which actually operates in the digestive tract of mammalians under the higher pH values. It sounds reasonable that this enzyme may operate under similar experimental conditions (high pH) to those reported in the present work. Furthermore, the ionic concentration created by the added NaOH solution could also help to stabilise the structure of the enzyme. Comparatively, most studied lipases are intracellular materials obtained from bacteria or fungi, so that optimum operation conditions employed in such cases are close to neutral pH conditions.

Covalent immobilization of PPL is carried out by using a methodology previously described in the heterogeneization of lipases [48–51], other enzymes like phosphatase [52] or glucose oxidase [53], as well as homogeneous organometallic complexes [54,55]. Taking into account the excellent results obtained in the immobilization of these enzymes, we have extended the possibilities of this methodology to be applied to the covalent attachment of PPL for use as an economically viable biocatalyst for the production of a novel biofuel integrating glycerol into its composition.

The kinetic properties of PPL have been determined before and after covalent immobilization on amorphous $AlPO_4$, as inorganic supports. Covalent binding to an insoluble support is the most interesting enzyme immobilization methodology because it combines the high selectivity of enzymatic reactions with the chemical and mechanical properties of the support. Covalent immobilization on the external surface of a support material has also been proposed to decrease mass transfer limitations associated with several immobilization techniques, such as entrapment or adsorption in gels. In this respect, inorganic matrices have a number of advantages over organic ones: no swelling and no porosity changes occur with pH and there is excellent storage stability of enzymes [56]. In this regard, we have previously reported the use of amorphous $AlPO_4$ as a metal support [57], as well as for heterogeneous catalysts in the field of selective organic synthesis [58–60]. This amorphous material, tailored by a controlled sol-gel method that allows us to obtain a high surface area as well as a high

number of surface -OH groups, is a very adequate support component for the covalent attachment of enzymes, according to the results obtained in the immobilization of lipases, phosphatase and GOD enzymes [48–53] as well as homogeneous organometallic complex [54,55].

2. Results and Discussion

In this study, some kinetic properties of the covalently immobilized PPL enzyme have been obtained. Enzymatic activities of immobilized PPL on amorphous $AlPO_4$, an inorganic support, were determined as compared to free one, under different working conditions such as reaction temperature, reaction pH, oil/alcohol ratio and enzyme concentration. The effect of these different parameters in the preparation of biofuels by ethanolysis of sunflower oil was investigated in previous works using free PPL in order to optimise the reaction conditions [37–39].

Tables 1 and 2 summarise the results obtained employing the immobilized PPL compared to the free enzyme, in the transesterification reaction with a unique biocatalyst, constituted by immobilized PPL on $AlPO_4$. The same quantity of supported biocatalyst was used in each reaction. Thus, in addition to obtain specific information about the influence of certain parameters (pH, temperature, *etc*.) on the behaviour of the immobilized PPL, their ability to be reused may be assessed. The number of re-uses is an essential parameter to measure the efficiency of the immobilization of the PPL.

Table 1. Composition, yield, conversion and TOF of the biodiesel obtained in the transesterification reaction of sunflower oil with 0.01 g of free PPL and 0.038 g of immobilized PPL in 0.5 g of support with different ratios of oil/absolute ethanol working in standard condition pH = 12, and different reaction time and temperatures.

No.	Oil/Ethanol (mL/mL)	T (°C)	t (h)	FAEE (%)	MG + DG (%)	TG (%)	Yield (%)	Conv. (%)	TOF (mmol/h g_{PPL})
0[a]	12/6	40	10	57.7	34.2	8.1	57.7	91.9	57.68
0[b]	12/6	40	10	13.4	65.1	21.5	13.4	78.5	58.26
1	12/12	40	24	6.1	23.3	70.6	6.1	29.4	0.67
2	12/6	25	24	11.7	71.0	17.3	11.7	82.7	1.28
3	12/6	30	69	53.3	46.7	0	53.3	100	2.03
4	12/6	35	48	64.5	31.3	0	64.5	100	3.53
5	12/6	40	31	67.0	27.7	5.3	67.0	94.7	5.68
6	12/6	45	20	59.9	40.1	0	59.9	100	7.88
7	45/4	25	24	16.9	41.8	41.3	16.9	58.7	6.94
8	45/4	30	44	40.9	15.4	43.7	40.9	56.3	9.17
9	45/4	35	30	34.4	24.8	40.8	34.4	59.2	11.32
10	45/4	40	18	27.2	51.2	21.6	27.2	78.4	14.91
11	45/4	45	24	43.7	23.9	32.4	43.7	67.6	17.97
12	45/4	50	23	44.3	33.6	22.1	45.3	77.9	19.43

[a] Free Enzyme (0.01 g); [b] Free enzyme in the supernatant solution.

Table 2. Composition, yield, conversion and TOF of the biodiesel obtained in the transesterification reaction of waste cooking oil using 96% ethanol with the same biocatalyst used in Table 1: 0.038 g of immobilized PPL in 0.5 g of support with different ratios of oil/96% ethanol working in standard condition pH = 12, and different reaction time and temperatures.

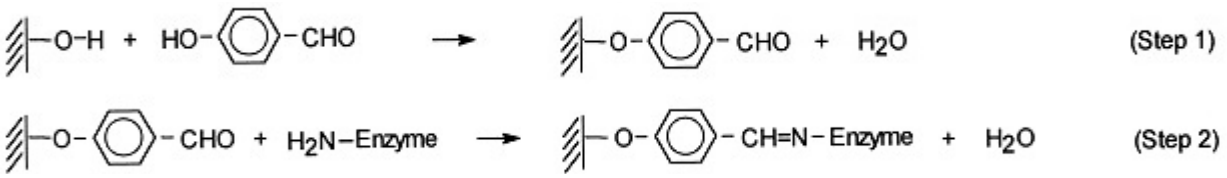
No.	Oil/EtOH (mL/mL)	T (°C)	t (h)	FAEE (%)	MG + DG (%)	TG (%)	Yield (%)	Conv. (%)	TOF (mmol/h g_{PPL})
13	45:7	40	21	37.3	36.0	26.7	37.3	73.3	17.53
14	45:7	45	20	50.2	48.3	1.5	50.2	98.5	24.77
15	45:7	50	24	51.4	40.9	7.7	51.4	92.3	21.13
16	36:6	35	28	24.1	75.9	0	24.1	100	6.80
	36:6	35	72	51.5	37.2	11.3	51.5	88.7	5.64
17	36:6	40	17	58.4	41.6	0	58.4	100	27.94
	36:6	40	25	54.3	41.2	4.5	54.3	95.5	17.15
	36:6	40	48	55.7	44.3	0	55.7	100	9.16
18	36:6	45	4	49.9	45.1	5.0	49.9	95.0	98.49
	36:6	40	11	54.8	40.6	4.6	54.8	95.4	39.33
19	36:6	50	19	32.6	65.1	2.3	32.6	97.7	13.55
	36:6	50	25	35.0	47.4	17.6	35.0	82.4	11.05
	36:6	50	46	56.4	34.7	8.9	56.4	91.1	9.67
	36:6	50	51	52.5	47.5	0	52.5	100	8.13
20	24:4	25	65	57.1	42.9	0	57.1	100	4.62
21	24:4	30	38	62.2	37.8	0	62.2	100	8.61
22	24:4	35	6	61.5	38.5	0	61.5	100	52.95
	24:4	35	10	61.9	38.1	0	61.9	100	32.58
	24:4	35	23	66.0	34.0	0	66.0	100	15.10
23	24:4	40	7	62.0	38.0	0	62.0	100	46.62
	24:4	40	14	61.1	38.9	0	61.1	100	22.97
24	24:4	45	9	58.7	41.3	0	58.7	100	34.33
25	24:4	50	7	63.7	36.3	0	63.7	100	47.89
	24:4	50	31	63.8	36.2	0	63.8	100	10.83

On the other hand, from the results in Table 1 we can determine the amount of enzyme covalently immobilized on the activated surface of the support in the immobilization process, step 2 in Scheme 2, where the support was functionalized with *p*-hydroxybenzaldehyde using a solid phase synthesis procedure following the published methodology [48,52,53].

In this sense, from the differences in enzymatic activity between the supernatant PPL, removed by washing (6 mL of ethanol) in the immobilization process (Table 1, No. 0^b) and those prepared in free form (Table 1, No. 0^a), operating under the same experimental conditions, the amount of covalently immobilized enzyme can be determined because the enzyme activity is normally proportional to the amount of free enzyme in solution. Thus, the quantity of immobilized enzyme can be determined from the differences in activity between the PPL in the supernatant and the standard quantity (0.01 g) of free PPL. The resulting solution (after the enzyme immobilisation) was filtered off, the reaction flask washed with 6 mL of ethanol and its catalytic activity was then tested. The filtrate gave a 13.4% yield

compared to the 57.7% yield obtained using the 0.01 g of free PPL, so that 0.0023 g of PPL was in the filtrate as free enzyme. The calculations 0.04 − 0.0023 = 0.038 g indicates that practically 94.3% of PPL was covalently immobilized on the activated $AlPO_4$.

Scheme 2. General scheme for covalent immobilization of the enzyme PPL through the ε-amino group of lysine residues. Activation of amorphous $AlPO_4$ support by microwave heating with *p*-hydroxybenzaldehyde (step 1) before covalent immobilization of the enzyme through the ε-amino group of lysine residues (step 2).



Therefore, a 94.3% of the enzyme was immobilized, in good agreement with previously reported results using this methodology [48,52,53]. Likewise, knowing the amount of immobilized PPL it is possible to obtain the corresponding Turnover Frequencies (TOF, mol h^{-1} $g_{PPL}{}^{-1}$), reaction rates calculated from the FAEE yield per unit of reaction time and weight of PPL employed. A good correlation was also obtained between the corresponding TOF values achieved with the filtrate (TOF = 58.3), as compared to the free PPL solution (TOF = 57.7).

The activities of the immobilized PPL were then investigated employing the same immobilized PPL in the ethanolysis of sunflower oil under different experimental parameters of interest, so that is assumed that in 25 consecutive reactions there is not any leaching of the immobilized PPL. In this respect, the calculation of corresponding TOF values in Tables 1 and 2 are carried out using the calculated values of supported biocatalyst (0.038 g of PPL) covalently immobilized on activated $AlPO_4$.

Table 1 shows experiences in which the molar ratios of oil/ethanol changes from 0.01/0.21 (Reaction No. 1), or 0.01/0.105 (reactions No. 2 to 6), where there is an excess of ethanol to ensure the complete transesterification, to 0.0375/0.07, (reactions No. 7 to 13) in which there is not enough ethanol to produce a complete transesterification, because only a 1:2 molar ratio of the reagents is not enough to complete the process. TOF values clearly indicate that an excess of ethanol, not only fails to give a 100% yield (which would lead to a standard biodiesel), but the transformation is even slower, similar to detected with the free enzymes [37].

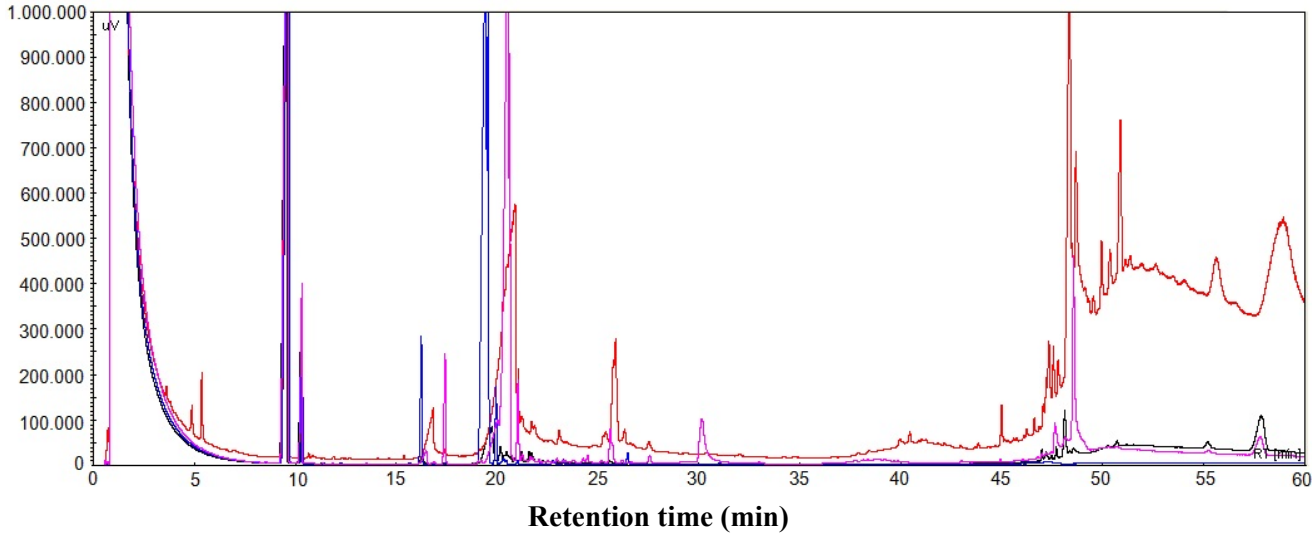
Table 2 indicates that, with independence of the quality of raw materials, even using waste cooking oil and 96% ethanol, when employed a 1:3 molar ratio of oil/ethanol, similar TOF values are obtained independent of the oil/PPL ratio applied in the processes. Given the stability of the immobilized enzymes (there is neither denaturation nor leaching), the transesterification stops for every reaction when it reaches the maximum theoretical yield of 66.6%, regardless of the reaction time that is needed. These results, similar to those obtained with free PPL [37–39] where it is not usual to exceed yields of 66%, corresponds to the transformation of one mole of TG in a mixture of two moles of FAEE and a mole of MG, consistent with a high selectivity towards the 1,3-positions in the processes described with most lipases, including PPL [61].

In this regard, the production of biodiesel using lipases needs to take into account such a regiospecific character [62,63]. In general, the challenge of fulfilling alcoholysis of triglycerides

involves long reaction times and gives conversions lower than 70 wt% in fatty acid methyl or ethyl esters [64,65]. A series of improvements in conversion levels and/or the use of methanol as alcohol to mimic the results of the base catalysed transesterification reaction are currently ongoing as a consequence of the present legal regulations for biodiesel (EN 14214). Reasonably good results are sometimes reported due to the observed 1,2-acyl migration in the monoglycerides [66–69]. Consequently, a major drawback that limits the widespread use of the enzymatic process is not only the high cost of the lipases but also the long reaction times needed to produce conventional biodiesel (100% FAME or FAEE) due to the limitations of the enzymatic method to induce the alcoholysis of the 2-fatty acid esters of glycerol. Therefore, although the enzymatic method is not the most appropriate for obtaining a standard biodiesel (100% FAME or FAEE), it is the best allowing the production of a biofuel composed of FAEE/MG mixture with a 2:1 molar ratio, which integrates the glycerol and allows a more efficient method for synthesis with the best environmentally benign methodology.

Furthermore, this biofuel that integrates glycerol as the monoglyceride not only improves the efficiency of the process but also presents physico-chemical properties similar to those of conventional biodiesel, obtained by alkaline catalysis, as it can be seen in Figure 1, where the gas-chromatograms of representative FAME and FAEE reactions and the corresponding MG reaction are collected. A representative sample of monoglycerides of sunflower oil is easily obtained. This is achieved by the substitution, in a conventional alcoholysis process, of methanol or ethanol by glycerol, and following identical standard experimental conditions.

Figure 1. Different chromatograms obtained in the alcoholysis of sunflower oil with methanol (FAME), ethanol (FAEE) and glycerol (monoacyl glycerols, MG), corresponding to blue, pink, and red, respectively. In black is the initial sunflower oil.



Here it can be seen that all chromatograms exhibit matching retention times (RT) for the internal standards at 10 min. This is a demonstration of the confidence of the method. By using commercial standards of FAME, FAEE and MG of palmitic and oleic acids it is possible to confirm that peaks with RT values between 16 and 18 min correspond to the derivatives of palmitic acid and that those between 19 and 21 min correspond to oleic acid derivatives. According to this, the RT values of the

methyl esters of fatty acids are very similar to those of the ethyl esters, and these are nearly coincident with the corresponding monoglycerides. Since the retention times of different derivatives of fatty acids are considered very closely related to the physico-chemical properties of these compounds, the great similarity of RT values obtained is a clear demonstration of the similarity among the rheological properties of different MG with their corresponding FAME or FAEE, which are crucial to allow its use as a fuel capable to substitute for petroleum products.

In this respect, it is also shown in Table 3 that representative biofuels integrating glycerine exhibited viscosity values similar to conventional biodiesel when methanol (3.9 cSt) or ethanol (6.6 cSt) are used in the alcoholysis process. Here it can also be seen how the viscosity is indeed highly dependent on the proportion of the TG in the sunflower oil or waste oil (considering their high viscosity values, 31.9 cSt or 44.6 cSt), and to a lesser extent, on the DG proportion. In Figure 1, we can see the presence of DG at high retention times, 40–60 min, which is not integrated, so that to determine DG + TG requires the use of internal standards. The presence of MG was also expected to have a similar influence on the viscosity of the biofuel that FAME or FAEE. In any case, by blending of these biofuels with diesel in a B20, the viscosity values are reduced enough to fulfil the required reference value (3.0–5.0 cSt). Thus, a B20 obtained from a 20% blend with commercial diesel, using a biofuel with υ = 15.0 cSt went to a 4.4 cSt value that allows its use in engines under current EN 14214 rules.

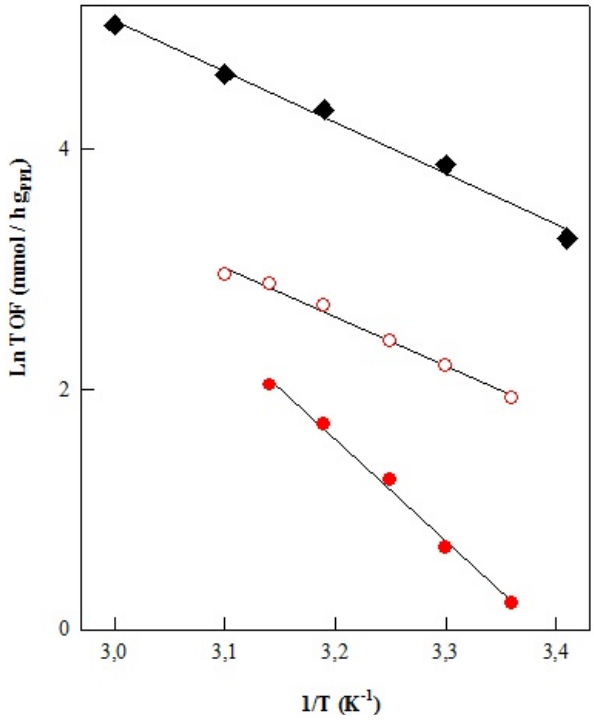
Table 3. Kinematic viscosity values, υ (cSt or mm^2/s) at 40 °C of various representative biofuel obtained under homogeneous alkaline or enzymatic catalysis as well as under heterogeneous catalysis, using both absolute and 96% ethanol, respectively. Kinematic viscosity of sunflower oil, commercial diesel and a B20 blend obtained from a biofuel (υ = 15.0) 20% and commercial diesel are also included.

Catalyst	Oil/Alcohol	FAE	MG + DG	Yield	Conv.	υ
NaOH	Waste/MeOH	95.7	4.3	95.7	100.0	3.9
KOH	Sunflower/ EtOH	94.8	5.2	94.8	100.0	6.6
PPL free	Sunflower/EtOH	55.7	44.2	55.7	100.0	6.9
-	Sunflower/EtOH	44.3	33.6	45.3	77.9	19.6
-	Waste/96% EtOH	54.3	41.2	54.3	95.5	23.4
-	Waste/96% EtOH	51.4	40.9	51.4	92.3	24.5
-	Waste/96% EtOH	66.0	31.0	66.0	100.0	19.7
-	Waste/96% EtOH	58.4	41.6	58.4	100.0	15.0
B20	-	-	-	-	-	4.4
Sunflower oil	-	-	-	-	-	31.9
Waste oil	-	-	-	-	-	44.6
Diesel	-	-	-	-	-	3.1

Taking into account that several experiments have been carried out using different oil/ethanol ratios, with the PPL enzyme, free and immobilized, in the alcoholisis reaction of sunflower oil (Table 1) and waste oils (Table 2), with both absolute and 96% ethanol, some additional information relative to the mechanism of heterogeneous enzymatic catalysis of PPL may be obtained, as well as an approximation to the effect of these variables in the mechanism, from the representation of Arrhenius, in Figures 2 and 3, that allows us calculate the values of activation energies and Arrhenius constants, from the slopes and

intercepts respectively, which are collected in Table 4. These values in some way quantify the effect of immobilization and experimental conditions have on the efficiency of PPL. Thus, the values of E_a inform about the efficiency of the active centers while the Arrhenius constant, LnA, report on the number of these active centers capable of promote the alcoholysis process. Similar values in both parameters could indicate similar reaction mechanisms between two processes.

Figure 2. Arrhenius representation of enzymatic activity obtained with free and immobilized PPL, in the transesterification of sunflower oil with absolute ethanol at pH = 12: (♦) Free PPL, EtOH; (●) Immob. PPL, Sunflower oil/EtOH 96% (12/6); (○) Immob. PPL, Sunflower oil/96% EtOH (45/4).



On the other hand, according to the Arrhenius equation, the higher values of E_a correspond to lower TOF values:

$$\mathrm{Ln(TOF)} = \mathrm{LnA} - E_a/RT \quad (1)$$

However, higher values of LnA indicate the existence of a high number of active sites where the reaction can be carried out. Interestingly, according to data in Table 4, the numerical values of E_a and LnA are very different for both free and immobilized PPL (under identical conditions; pH 12 and 2/1 oil/alcohol V:V ratio). Thus, the free and covalently immobilized PPL operates under a different reaction mechanism. Therefore, after immobilization, the number of active sites is increased (LnA is increased from 17.76 to 29.08). This could be explained as a consequence of the formation of a higher dispersion of the PPL on the solid surface after the covalent immobilization. However, the activity of these active sites is clearly decreased, as a consequence of the increased values of E_a obtained after the immobilization (E_a is increased from 8.4 to 16.8). This behavior could be explained as a consequence of steric effects on immobilized active sites, where the access of reactants could be more restricted than in a free PPL.

Figure 3. Arrhenius representation of enzymatic activity obtained with free and immobilized PPL, in the transesterification of used oil with 96% ethanol at pH = 12: (♦) Free PPL, EtOH; (●) Immob. PPL, waste oil/96% EtOH (45/7); (○) Immob. PPL, waste oil/96% EtOH (36/6); (▲) Immob. PPL, waste oil/96% EtOH (24/4).

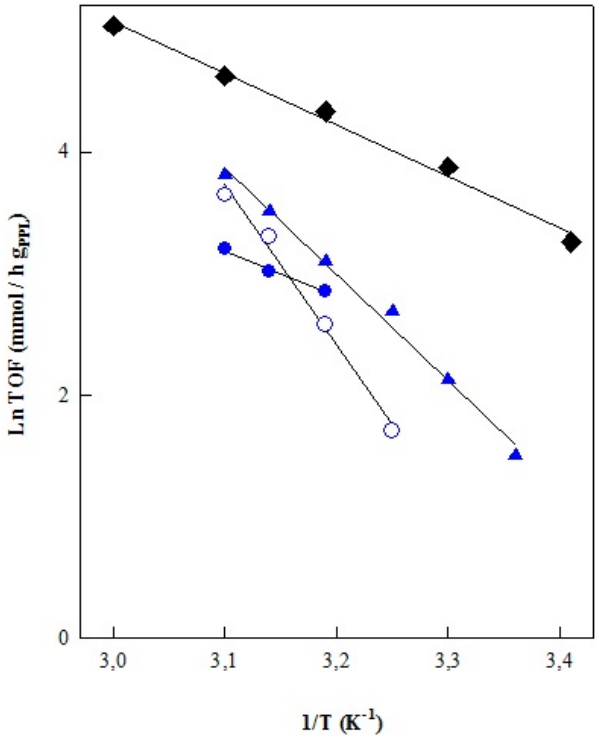


Table 4. Activation Energy values E_a (Kcal/mol) and preexponential factor LnA (h^{-1}), obtained in the ethanolisis reaction of oils with free and immobilized at pH = 12.

PPL Enzyme	Oil/Vol (mL)	Acohol/Vol (mL)	E_a (Kcal/mol)	LnA (h^{-1})	r^2
Free	Sunflower/12	EtOH abs./6	8.40 ± 0.24	17.76 ± 0.76	0.99
Immobilized	Sunflower/12	EtOH abs./6	16.80 ± 0.41	29.08 ± 1.33	0.99
Immobilized	Sunflower/45	EtOH abs./4	8.12 ± 0.20	15.89 ± 0.65	0.99
Immobilized	Waste/45	96% EtOH/7	7.65 ± 0.39	15.33 ± 1.22	1
Immobilized	Waste/36	96% EtOH/6	26.22 ± 0.89	45.28 ± 2.83	0.99
Immobilized	Waste/24	96% EtOH/4	17.42 ± 0.39	31.49 ± 1.26	0.99

On the other hand, a variation of the oil/alcohol molar ratio deeply changed the values of E_a and LnA for the immobilized PPL. Smaller quantities of alcohol provided a greater number of active sites participant in the enzymatic process (greater LnA) and improved the efficiency (greater E_a), therefore promoting the alcoholysis. Thus, when operating at higher concentrations of oil regarding to the enzyme (whether it is used waste oil or sunflower oil), the mechanism of immobilized PPL seems to be closer to that of free PPL, according to values of Ea and LnA in Table 4 (see white dotted line in Figure 2 and black dotted line in Figure 3). This behavior is practically the opposite of an alcoholysis process developed under alkaline homogeneous catalysis.

Of note was also the enzyme stability and recyclability after the covalent immobilization. Although the efficiency was reduced compared to the free form, the immobilisation of the PPL guaranteed the lifespan of the lipases. The free PPL was found to be completely deactivated in 48 h, whereas the

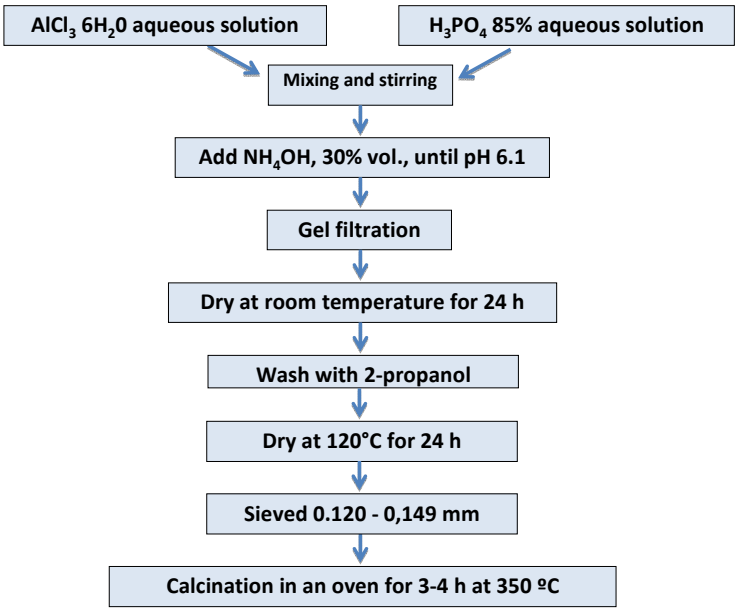
immobilized enzyme was active for several weeks, even after successive reuses (Tables 1 and 2), preserving over 90% of the initial activity, as can be obtained from the comparison of reactions 2 and 3 wit 21 and 22, respectively.

3. Experimental Section

3.1. Synthesis and Surface Functionalization of $AlPO_4$ Used as Support

The amorphous aluminium phosphate ($AlPO_4$) used as support for the covalent immobilization of PPL was obtained according to the sol-gel method used elsewhere to obtain other metal phosphates, metal supports and heterogeneous catalysts in different organic synthesis reactions [56–60], as well as in the covalent immobilization of several enzymes and organometallic complexes [48,52–55]. Thus, this amorphous $AlPO_4$ (Al/P = 1) employed as PPL support was specially developed to obtain solids with high surface area and high density of Brönsted acid sites, what making them particularly suitable for the surface functionalization with some organic molecules, through the reaction of the surface acid sites with amino or hydroxyl groups of these activating molecules [48,52–55]. In this way, amorphous $AlPO_4$ was prepared by precipitation from aqueous solutions of $AlCl_3 \cdot 6H_2O$ and H_3PO_4 (85 wt%) at pH = 6.1 at the "precipitation end point", with ammonium hydroxide solution, according to the chart flow outlined in Figure 4. The solid obtained after filtration was then washed with isopropyl alcohol and dried at 120 °C for 24 h. In the present case the resulting powder was calcined for 3 h in an electric muffle furnace at 350 °C and screened to a particle size at 0.120–0.149 mm, before its use as support for covalent immobilization of PPL.

Figure 4. Chart flow of the sol-gel preparation schedule for amorphous aluminium phosphate support.



Amorphous $AlPO_4$ obtained through this protocol synthesis exhibits a high surface area (S_{BET} = 211 $m^2\ g^{-1}$), a high number of Brönsted acid sites (249 µmol g^{-1}) as well as a high number of basic sites (352 µmol g^{-1}). The surface area, S_{BET} in $m^2\ g^{-1}$ was calculated by the BET method, from

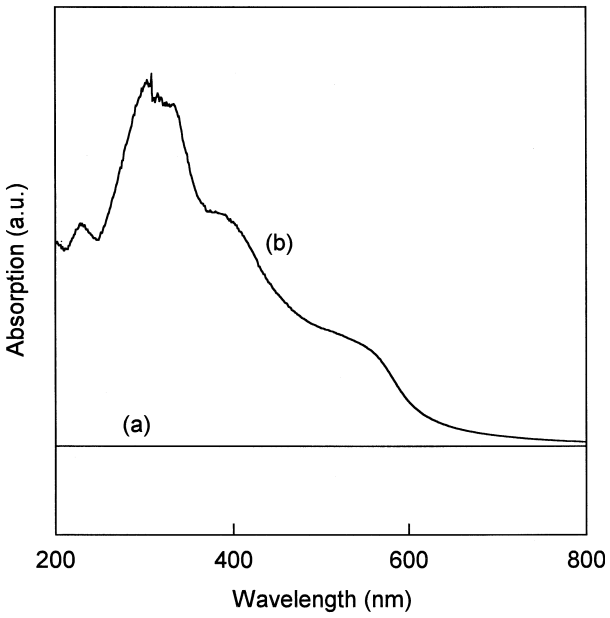
nitrogen adsorption-desorption isotherms, determined at liquid nitrogen temperature by using a Micromeritics ASAP-2000 instrument. Prior to measurements, all samples were degassed at 200 °C to 0.1 Pa. Values of pore volume, V = 0.72 cm^3 g^{-1}; and mean pore diameter, d = 12.8 nm were also obtained. The surface acid–base properties of amorphous $AlPO_4$ was determined by a spectrophotometric method that allows titration of the amount (in μmol g^{-1}) of amounts of organic probes irreversible adsorbed from cyclohexane solutions, such as pyridine (PY, pKa = 5.25), 2,6-dimethylpyridine (DMPY, pKa = 6.99), and benzoic acid (BA, pKa = 4.19, employed as titrant molecules of acid and basic sites, respectively [52–60].

The support was functionalized with *p*-hydroxybenzaldehyde using a solid phase synthesis procedure, according to step 1 in Scheme 1, following the published methodology [48,52,53]. Activation of the $AlPO_4$ support surface was initiated by covalent anchoring of *p*-hydroxybenzaldehyde, a functionalized linker, through the reaction of its OH phenolic group with -OH Brönsted acid groups on support surface [48–54]. The phosphoester bond was obtained by a microwave heating reaction (15 min at 380 W) of the support (20 g) and *p*-hydroxybenzaldehyde (2 g). The activation process is carried out therefore in one step, in a quick and clean way in a conventional microwave, being possible to perform the covalent immobilization of the PPL in the same reaction flask, after washing the solid with acetone to clean the activated solid from unreacted aldehyde (step 2 in Scheme 1). The efficacy of immobilization of the process is confirmed by Visible-Ultraviolet/Diffuse Reflectance experiments shown in Figure 5. Spectra were recorded on a Varian Cary 1E UV-Visible Diffuse Reflectance Spectrophotometer (Varian Australia Pty Ltd, Victoria, Australia), between 200 and 800 nm wavelengths. We can check the change in the surface of the inorganic solid used as support in the treatment that lead to the functionalization with superficial aromatic aldehyde groups able to react and immobilize the enzyme proteins, Scheme 1.

3.2. PPL Immobilization and Enzymatic Activity

The immobilization of PPL is carried out at room temperature, by introducing the functionalized inorganic solid (0.5 g) with the PPL (0.04 g) in a reaction flask (50 mL) with ethanol (6 mL), stirring and keeping in a refrigerator for 24 h, stirring occasionally every three or 4 h to get the covalent interaction of the ε-amino group of the lysine residues of the PPL with the aldehyde groups of linkers (step 2 in Scheme 1). Finally, prior to its use, ethanol (6 mL) was added to the mixture and the solid, with the immobilized PPL, was then separated by filtration and centrifugation from the solution containing the remaining non-immobilized lipase. The catalytic activity of this dissolution is proportional to the amount of PPL dissolved. Thus, we can easily determine the quantity of PPL which has not been immobilized, remaining in supernatant dissolution. The comparison of this value with the activity of immobilized and free PPL enzymes will allow us to determine the amount of immobilized enzyme, and its efficacy [48–53].

Figure 5. Visible-ultraviolet/diffuse reflectance spectra of different samples obtained in different steps in Scheme 2: (**a**) $AlPO_4$ support and (**b**) 4-hydroxybenzaldehyde on support after microwave heating (step 1).



3.3. Ethanolysis Reactions

The ethanolysis reactions were performed in a 50 mL round bottom flask under continuous stirring at controlled temperature (25–50 °C) varying the pH values in the 8–12 range. The various pH environments were achieved by adding different quantities of aqueous solutions of NaOH 10N (0.1–0.01 mL). The reaction mixture comprised sunflower oil (9.4 g, 12 mL, 0.01 mol), a variable oil/alcohol volume ratio and solid containing the immobilized PPL (0.5 g). Free PPL (0.01 g) was also used as reference, to determine the efficiency and amount of immobilized enzyme. A commercial crude PPL (Type II, L3126, Sigma-Aldrich, St. Louis, MO, USA, 100−400 units/mg protein, using olive oil with 30 min incubation and 30−90 units/mg protein using triacetin), sunflower oil for food use and used cooking oil obtained from a local company collecting this waste product. Ethanol (Panreac, Barcelona, Spain, 99% and Alcoholes del Sur, Córdoba, Spain, 96%) were employed in the reactions. Waste cooking oil was provided by a local collector from domestic consumers. It was filtered before its use but no additional purification treatment was performed. This oil presented a density of 0.898 g/mL, a water content of 0.7 wt%, determined by Karl Fisher standard method, an acid value of 0.82 mg of KOH/g of oil, and an iodine value of 120.5. According to GC analysis, the fatty acid composition is close similar to sunflower oil, with oleic (C18:1 9c) and linoleic (C18:2 9c 12 c) acids as the main fatty acid components.

3.4. Compositional Analysis of Reaction Products by Gas Chromatography

Samples were periodically withdrawn at different reaction times (6–48 h) and quantified using a Varian 430 GC gas chromatograph (Varian, Inc., Palo Alto, CA, USA) equipped with a flame ionization detector (FID) at 450 °C, splitless injection at 350 °C and connected to a capillary column HT5, 0.1 UM (25 m × 0.32 mm, SGE Supelco, St. Louis, MO, USA). Cetane (*n*-hexadecane)

was employed as internal standard to quantify the content of ethyl esters and glycerides (-mono, di and triglycerides) with the help of some commercial standard fatty acid esters, respectively. As carrier gas nitrogen is used, with a flow of 1.5 mL/min, it has been applied a heating ramp from 90 °C to 200 °C at a rate of 7 °C/min, followed by another ramp from 200 °C to 360 °C at a rate of 15 °C/min, maintaining the temperature of the oven at 360 °C for 10 min.

The results are expressed as relative quantities of the corresponding Fatty Acid Ethyl Esters (FAEE) and the sum of the quantities of MG and diglycerides (DG). The yield refers to the relative amount of FAEE produced (%). The conversion includes the total amount (%) of triglyceride transformed (FAEE + MG + DG). The reaction rates and turnover frequencies (TOF, mol h^{-1} $g_{PPL}{}^{-1}$) were calculated from the yield, considering the amount of FAEE generated per unit of time of reaction and weight of PPL employed. The blank reaction in the presence of the highest quantity of solution of NaOH was performed to rule out a potential contribution from the homogeneous catalysed reaction promoted by the present experimental conditions. Less than 15% conversion of the starting material was found under these conditions, implying the production of the biofuel was caused by the enzyme.

3.5. Viscosity Measurements

The viscosity was determined in a Cannon-Fenske Routine Viscometer 33200 capillary viscometer, size 200 (Cannon Instrument Company, State College, PA, USA). This is based on determining the time needed for a given volume of fluid passing between two points marked on the instrument. It correlates to the speed reduction suffered by the flow of liquid, as a result of internal friction of its molecules, depending on their viscosity. From the flow time, *t*, in seconds, the kinematic viscosity (υ, centistokes, cSt) can be obtained from the equation: $\upsilon t = C$, where C is the constant calibration of the measuring system in cSt s, which is given by the manufacturer (0.10698 mm^2 s^{-1}, at 40 °C) and *t* is the flow time in seconds. The kinematic viscosity also represents the ratio between the dynamic viscosity and the density (ρ, $\upsilon = \eta/\rho$).

4. Conclusions

Although the efficiency of PPL is remarkably reduced in the production of a conventional biodiesel (100% FAEE) due to the 1,3-regiospecific character of PPL, this weakness becomes a strength when we consider an alternative objective consisting in obtaining a biofuel that keeps glycerol as a monoglyceride molecule, because it presents physico-chemical properties similar or even better than conventional biodiesel as well as all kinds of benefits related to the quality of the biofuel and the fabrication process. Thus, the stereoselective character of PPL and most lipases is what permits one to obtain this new biofuel that incorporates glycerol as MG molecules, by the 1,3-selective ethanolysis of TG, minimising the waste production and improving the reaction conversion under greener conditions, so that it can play an advantageous role, compared to the conventional alkaline catalysed process.

From the results it can be concluded that milder reaction conditions were employed (temperatures in the range 30–45 °C and 2/1 volume ratio of oil/ethanol) and a cleaner biofuel was obtained. The atom efficiency is also improved to 100% because the total number of atoms involved in the reaction

(as reactants) becomes part of the final mixture that constitutes the biofuel. Besides, ethanol not spent in the enzymatic process remains in the reaction mixture in such a way that after the reaction the product blend obtained can be directly used as a fuel. In this respect, very recent studies [70–72] have proven that blends of diesel fuel and ethanol with biodiesel produced a slightly less maximum power output than regular diesel. No significant difference in the emissions of CO_2, CO, and NO_x between regular diesel and biodiesel, ethanol and diesel blends was observed, but the use of these blends resulted in a reduction of particulate matter. Consequently such blends can be used in a DI diesel engine without any modification taking into account the limited changes obtained respect to the use of pure diesel. Thus, the ecodiesel expression is currently ascribed to whichever blend of fatty acid alkyl ester with ethanol, alone or with any proportion of diesel fuel [70–73].

The covalent immobilized PPL enzyme was highly stable and although the efficiency was reduced compared to the free enzyme, the biocatalyst can easily be reused repeatedly (25 times) almost preserving the initial catalytic activity. This indicates the capability of the phosphamide bond to get efficient links between different organic molecules and the external surface of an amorphous inorganic solid such as $AlPO_4$. Thus, the high stability of phosphamide bonds plays a crucial role in the covalent immobilization of PPL biomolecules. Consequently, through the phosphamide bond a versatile procedure to obtain hybrid organic-inorganic molecules able to produce the covalent attachment to inorganic supports of many different organic molecules, including biomolecules like lipase enzymes, is achieved. Thus, within the same general schemes currently used with silanized SiO_2 [74–77], these organic molecules can be modified following the experimental methodology of the solid-phase synthesis, to obtain adequate ligands linked to the solid surfaces [48–55].

Finally we have that, although this new biofuel is outside the EN 14214 standard, several papers have demonstrated that monoglycerides enhance the lubricity of fuels respect to standard biodiesel [41–45]. Thus, its incorporation to the market of biofuels has not currently any technical limitation. In this respect, the results obtained let to open also the possibility to use bioethanol instead of methanol, a refinery product from petroleum, as well as they also expand the possibility to use not only PPL but also other 1,3-selective lipases [40] which are only able to give as much as 66%–70% of conversion due to the difficulty in producing the alcoholysis of the 2-fatty acid esters of glycerol. Additional studies [78] are currently being conducted to optimize the conditions for covalent immobilization of PPL to produce this new biofuel.

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Conflict of Interest

The authors declare no conflict of interest.

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